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microRNA-10b enhances pancreatic cancer cell invasion by suppressing TIP30 expression and promoting EGF and TGF-β actions

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Increased microRNA-10b (miR-10b) expression in the cancer cells in pancreatic ductal adenocarcinoma (PDAC) is a marker of disease aggressiveness. In the present study, we determined that plasma miR-10b levels are significantly increased in PDAC patients by comparison with normal controls. By gene profiling, we identified potential targets downregulated by miR-10b, including Tat-interacting protein 30 (TIP30). Immunoblotting and luciferase reporter assays confirmed that TIP30 was a direct miR-10b target. Downregulation of TIP30 by miR-10b or siRNA-mediated silencing of TIP30 enhanced epidermal growth factor (EGF)-dependent invasion. The actions of miR-10b were abrogated by expressing a modified TIP30 cDNA resistant to miR-10b. EGF-induced EGF receptor (EGFR) tyrosine phosphorylation and extracellular signal–regulated kinase phosphorylation were enhanced by miR-10b, and these effects were mimicked by TIP30 silencing. The actions of EGF in the presence of miR-10b were blocked by EGFR kinase inhibition with erlotinib and by dual inhibition of PI3K (phosphatidylinositol 3′-kinase) and MEK. Moreover, miR-10b, EGF and transforming growth factor-beta (TGF-β) combined to markedly increase cell invasion, and this effect was blocked by the combination of erlotinib and SB505124, a type I TGF-β receptor inhibitor. miR-10b also enhanced the stimulatory effects of EGF and TGF-β on cell migration and epithelial–mesenchymal transition (EMT) and decreased the expression of RAP2A, EPHB2, KLF4 and NF1. Moreover, miR-10b overexpression accelerated pancreatic cancer cell (PCC) proliferation and tumor growth in an orthotopic model. Thus, plasma miR-10b levels may serve as a diagnostic marker in PDAC, whereas intra-tumoral miR-10b promotes PCC proliferation and invasion by suppressing TIP30, which enhances EGFR signaling, facilitates EGF–TGF-β cross-talk and enhances the expression of EMT-promoting genes, whereas decreasing the expression of several metastasis-suppressing genes. Therefore, therapeutic targeting of miR-10b in PDAC may interrupt growth-promoting deleterious EGF–TGF-β interactions and antagonize the metastatic process at various levels.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in the United States with a median survival of 6 months and a 5-year survival rate of 6%.[1] As there are no effective modalities for early detection, most PDAC patients present with locally invasive and/or metastatic disease and are not candidates for resection.[2] Histologically, PDAC is characterized by regions of acinar-to-ductal metaplasia, pancreatic intra-epithelial neoplasia, cancer cells forming ductal-like structures, inflammatory infiltrates and an extensive desmoplastic reaction that interferes with effective drug delivery into the pancreatic tumor mass.[3–5] At the molecular level, there is a high frequency of major driver mutations in key regulatory genes, including *KRAS*, *TP53*, *CDKN2A* and *SMAD4*, an abundance of low-frequency driver mutations and aberrant activation of multiple signaling pathways as a consequence of the overexpression of multiple tyrosine kinase receptors, such as the epidermal growth factor (EGF) receptor (EGFR) and its ligands, and excessive production of transforming growth factor-beta (TGF-β) isoforms.[6,7]

EGFR homodimerization and heterodimerization with other EGFR family members leads to enhanced tyrosine kinase activity of the receptor complexes and activation of multiple signaling pathways, such as mitogen-activated protein kinase (MAPK), p38 MAPK, phosphatidylinositol 3′-kinase (PI3K), Src, Crk and Nck.[8,9] By contrast, TGF-β activates serine-threonine receptor kinases, which act through canonical, Smad-dependent signaling as well as non-canonical, Smad-independent signaling, and generally exert paracrine effects on the tumor microenvironment in PDAC.[10–12] However, TGF-β can also directly enhance the invasion of pancreatic cancer cells (PCCs) and stimulate PCC proliferation,[13] indicating that EGF–TGF-β interactions are important with respect to cell-autonomous effects in PDAC.

MicroRNAs (miRs) constitute a class of 18–25 nucleotide non-coding RNAs that target specific mRNA moieties for translational repression or degradation.[14] Expression of miRs is often deregulated in cancer, and miRs participate in the regulation of many important biological processes, including cell survival, proliferation, migration, invasion and metastasis.[15,16] Several miRs are overexpressed in PDAC, including miR-10b, miR-21,

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miR-196a, miR-203, miR-155, miR-210 and miR-221.[17–22] Moreover, miR-10b is one of the most frequently upregulated miRs in PDAC,[18,22] and high miR-10b levels in the cancer cells in PDAC are associated with decreased therapeutic response to multimodality neoadjuvant therapy, shorter time to metastasis, decreased overall patient survival and increased PCC invasiveness.[22–24] However, it is not clear how miR-10b promotes PCC invasion in PDAC.

In the present study, we determined that miR-10b enhanced EGF-induced invasion, EGFR phosphorylation and extracellular signal–regulated kinase 1 (ERK1)/2 activation in PCCs, whereas attenuating EGFR degradation and that these effects were due to miR-10b-induced downregulation of Tat-interacting protein 30 (TIP30). Moreover, the combination of high levels of miR-10b, EGF and TGF-β markedly enhanced cell invasion and altered gene expression in a manner that was consistent with epithelial–mesenchymal transition (EMT) induction. Given that miR-10b, EGFR and TGF-β are often overexpressed in PDAC,[22–26] these observations suggest that targeting miR-10b may serve to suppress metastases and to interrupt deleterious EGF–TGF-β interactions in PDAC.

RESULTS

miR-10b is expressed at high levels in the cancer cells in PDAC,[18,22] and several studies have implicated miR-10b in cancer metastasis.[27,28] To confirm that miR-10b expression is increased in PDAC, we took advantage of the known resistance of miRs to degradation in the circulation and assayed miR-10b levels in the plasma of 20 normal controls, 5 patients with chronic pancreatitis and 17 PDAC patients. miR-10b levels were increased 575-fold in PDAC patients by comparison with the corresponding levels in either normal controls ($P<0.001$) or patients with chronic pancreatitis (Figure 1a; $P<0.001$).

Given that the metastatic process is preceded by invasion, we sought to identify invasion-associated miR-10b targets that could lead to enhanced metastasis in PDAC. Accordingly, microarray studies were conducted using RNA from PANC-1 cells transfected with a miR-10b precursor. Fifty-five genes were downregulated by at least 40% upon miR-10b overexpression (Supplementary Table S1), and three of these genes (*RAP2A*, *EPHB2* and *TIP30*) affect pathways that have the potential to suppress cancer cell invasion. Quantitative reverse transcription PCR (qRT–PCR) confirmed that the mRNA levels of all three genes were significantly decreased in PANC-1 cells expressing high levels of miR-10b compared with respective controls (Figure 1b). *In silico* analysis with miRanda, PicTar and TargetScan computational tools for miRNA target prediction confirmed that *TIP30*, *RAP2A* and *EPHB2* were potential miR-10b targets.

TIP30, also known as HIV-1 Tat interactive protein 2 (HTATIP2) or *CC3*, was of particular interest, because it suppresses metastases in

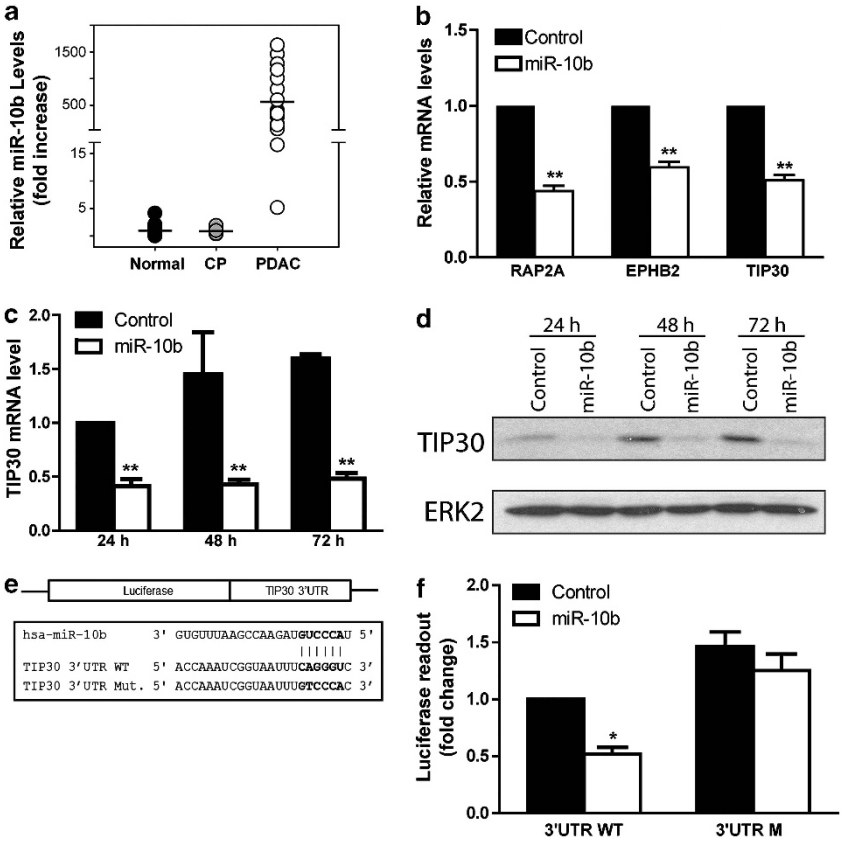

Figure 1. miR-10b targets. (**a**) Plasma miR-10b levels. Compared with plasma from normal controls ($n=20$; closed circles) and chronic pancreatitis patients (CP; $n=5$; gray circles), miR-10b levels are significantly elevated ($P<.001$) in the plasma of PDAC patients ($n=18$; open circles). Horizontal bars denote mean expression levels. (**b**) Gene expression. qRT–PCR of indicated mRNAs in PANC-1 cells transfected with control (solid bars) or precursor miR-10b (open bars) confirmed the array results. (**c**) TIP30 levels. qRT–PCR was used to determine TIP30 mRNA levels in PANC-1 cells transfected with control (solid bars) or precursor miR-10b (open bars) for the indicated times. (**d**) TIP30 immunoblotting. A highly specific anti-TIP30 antibody was used in immunoblotting analysis, revealing a marked decrease in TIP30 protein levels in the presence of miR-10b by comparison with control. (**e**) Luciferase reporter constructs. The reporter constructs encoded a wild-type (WT) TIP30 3′UTR or a mutant (M) TIP30 3′UTR in which the binding site was replaced by the indicated nucleotides. (**f**) Luciferase readout. PANC-1 cells were transfected with control (solid bars) or precursor miR-10b (open bars) for 20 h together with WT or M TIP30 3′UTR luciferase constructs. Data in panels **b**, **c**, and **f**, are the means ± s.e.m. from three experiments, analyzed by two-way ANOVA followed by Bonferroni's adjustment. $^{*}P<0.01$ and $^{**}P<0.001$ compared with its respective controls.

lung, breast and hepatocellular cancers[29–31] and regulates EGF-induced EGFR endocytosis.[32] Given the important role of EGFR in PDAC,[25,33] we sought to confirm that miR-10b downregulates TIP30 mRNA and protein levels. Accordingly, qRT–PCR and immunoblotting were conducted using RNA and protein extracted from miR-10b-overexpressing PANC-1 cells. Increased miR-10b expression led to decreased TIP30 mRNA (Figure 1c) and protein (Figure 1d) levels at 24, 48 and 72 h post transfection. A similar decrease in TIP30 protein levels was seen in ASPC-1, COLO-357 and T3M4 human PCCs following transfection with miR-10b precursor (Supplementary Figure S1a), indicating that this downregulation was not unique to PANC-1 cells. Moreover, transfection with the miR-10a precursor, which shares the same sequence with miR-10b except for a difference of one nucleotide in the non-seed region,[34] resulted in variable but reproducible decreases in TIP30 levels in all the four PCCs (Supplementary Figure S1a).

To confirm that TIP30 is a direct target of miR-10b, we used a TIP30 3′ untranslated region (UTR) luciferase construct, and a mutant construct in which 6 nucleotides in the potential binding site were replaced (Figure 1e). Co-transfection of PANC-1 cells with the TIP30 3′UTR construct and miR-10b precursor caused a 50% decrease in luciferase activity compared with the negative control, and this suppression of TIP30 expression was completely reversed by the six-nucleotide substitution in the core binding site (Figure 1f).

EGF and TGF-β promote PCC invasion.[12] To determine whether miR-10b enhances human PCC invasion and whether it modulates the actions of either EGF or TGF-β, miR-10b precursor was transfected into two human PCCs. In PANC-1 and COLO-357 cells, EGF (1 nM) or TGF-β1 (0.5 nM) enhanced invasion, and their combined action was greater than that of either growth factor alone (Figures 2a and b). Overexpression of miR-10b increased invasion in PANC-1 cells and enhanced EGF-mediated invasion in both PCCs and TGF-β1-mediated invasion in COLO-357 cells. Moreover, the combination of both growth factors exerted a significantly ($P<0.005$) greater stimulatory effect on invasion in cells with high miR-10b levels (Figures 2a and b), which is potentially important clinically given that miR-10b, EGFR and TGF-β are all overexpressed in PDAC.[22–26]

To determine whether TIP30 knockdown mimics miR-10b actions on PCC invasion, PANC-1 cells were transfected with two siRNAs targeting TIP30. TIP30 silencing by either siRNA reduced TIP30 levels (Supplementary Figure S1b) and increased EGF-stimulated invasion (Figure 2c), indicating that TIP30 is a negative regulator of PCC invasion. To confirm that TIP30 downregulation was essential for miR-10b-induced stimulation of cell invasion, we used the pCMV-SPORT6-TIP30 vector, which encodes a TIP30 cDNA that is not regulated by miR-10b due to a mutated 3′UTR binding site. Experiments were carried out in the presence of 1 nM EGF and in the absence or presence of transfected miR-10b using cells expressing empty vector or the pCMV-SPORT6-TIP30 vector. This experimental design allowed for the specific evaluation of the consequence of expressing a TIP30 that was resistant to miR-10b downregulation. When pCMV-SPORT6-TIP30 was transfected into PANC-1 cells, the stimulatory effect of miR-10b overexpression on EGF-induced invasion was completely abrogated (Figure 2d). Thus, TIP30 is a functional target of miR-10b whose downregulation promotes EGF-induced invasion.

Invasion is often associated with enhanced cancer cell motility. Therefore, we next sought to determine whether miR-10b modulated PCC migration. In COLO-357 cells, miR-10b, EGF (1 nM), TGF-β1 (0.5 nM) or the combination of EGF and TGF-β1 also increased cell migration (Figure 2e). Moreover, in the presence of miR-10b, the combined effect of EGF and TGF-β1 on migration was significantly greater than with either growth factor alone (Figure 2e). By contrast, in PANC-1 cells EGF enhanced migration only following miR-10b transfection, whereas TGF-β1 and the combination of EGF and TGF-β1 significantly increased migration in control cells, and these effects were enhanced in the presence of miR-10b (Figure 2f).

Specific inhibitors were used next to confirm that EGF and TGF-β acted through their respective receptors with respect to their individual and combined stimulatory effects on invasion in the presence of miR-10b. The EGFR inhibitor erlotinib (2 μM) completely blocked EGF-induced invasion in the presence of miR-10b in both the COLO-357 and PANC-1 cells (Figures 3a and b). Although the PI3K inhibitor LY294002 (10 μM) and the MEK inhibitor UO126 (1 μM) partially blocked this effect, the combination of LY294002 and UO126 were as effective as erlotinib in suppressing EGF-mediated invasion (Figures 3a and b). Moreover, miR-10b's effects on combined EGF–TGF-β1 actions on invasion were markedly attenuated by EGFR kinase inhibition with erlotinib and TβRI kinase inhibition with SB505124 and were abrogated by their combined actions (Figures 3c and d), indicating that EGF and TGF-β1 act through their respective receptors to cross-talk and enhance invasion.

TIP30 is a crucial component of the complex that regulates endocytotic trafficking of EGFR in breast and liver cancer cells and may act to prolong downstream signaling.[31,32] We therefore sought to determine whether miR-10b modulates EGFR expression and signaling in PANC-1 cells. In control-transfected cells, EGF caused a gradual decrease in EGFR protein levels, and this effect was attenuated in miR-10b-overexpressing cells (Figure 3e) but was not associated with any alteration in EGFR mRNA levels (not shown). EGF also rapidly increased EGFR (Tyr 1148) and ERK1/2 phosphorylation (Thr-202/Tyr-204), and both of these effects were enhanced by miR-10b (Figure 3e). By contrast, EGF-induced AKT phosphorylation (Ser 473) and p38 MAPK phosphorylation (Thr-180/Tyr-182) were not altered by miR-10b (Figure 3e).

To assess whether downregulation of TIP30 alone is sufficient to modulate EGFR signaling in PCCs, TIP30 levels were suppressed with siRNA 6 (Supplementary Figure S1b). TIP30 knockdown attenuated EGF-induced EGFR downregulation and enhanced EGFR and ERK1/2 phosphorylation without altering p38 MAPK phosphorylation (Figure 3f). In contrast to the actions of miR-10b, siRNA targeting of TIP30 enhanced EGF-induced AKT phosphorylation (Figure 3f), suggesting that TIP30 may suppress pro-survival signals independently of its actions on EGFR downregulation.

Cancer cell invasion is often associated with enhanced EMT, and TGF-β1 induces EMT in PCCs.[35] Accordingly, we next sought to determine whether miR-10b modulated the effects of TGF-β and EGF on the expression of EMT-associated genes (Figure 4). In both the COLO-357 and PANC-1 cells, miR-10b significantly decreased E-cadherin levels, and this effect was most pronounced in the presence of the combination of TGF-β1 and EGF (Figure 4a). In both the cell lines, EGF plus TGF-β1 increased N-cadherin and vimentin expression but only in the presence of high miR-10b levels (Figures 4b and c). Moreover, in the presence of miR-10b, N-cadherin expression was increased by TGF-β1 alone in COLO-357 cells and by EGF alone in PANC-1 cells, where EGF plus TGF-β1 also increased N-cadherin in the absence of added miR-10b (Figure 4b). Snail and Zeb1 expression was increased in COLO-357 cells in response to either or both the growth factors in the presence of miR-10b, as well as in the presence of both the growth factors without added miR-10b (Figures 4d and e). By contrast, PANC-1 cells exhibited increased Snail levels only in response to both the growth factors, and this effect was increased slightly by miR-10b (Figure 4d). Increases in Zeb1 expression in PANC-1 cells paralleled those observed in COLO-357 cells, except that TGF-β1 was effective even in the absence of added miR-10b (Figure 4e).

We next examined the effects of miR-10b on the expression of several known miR-10b target genes,[27,36–40] *HOXD10*, *TIAM1*, *KLF4*, *SDC1* and *NF1* in AsPC-1, COLO-357 and PANC-1 cells. By qRT–PCR,

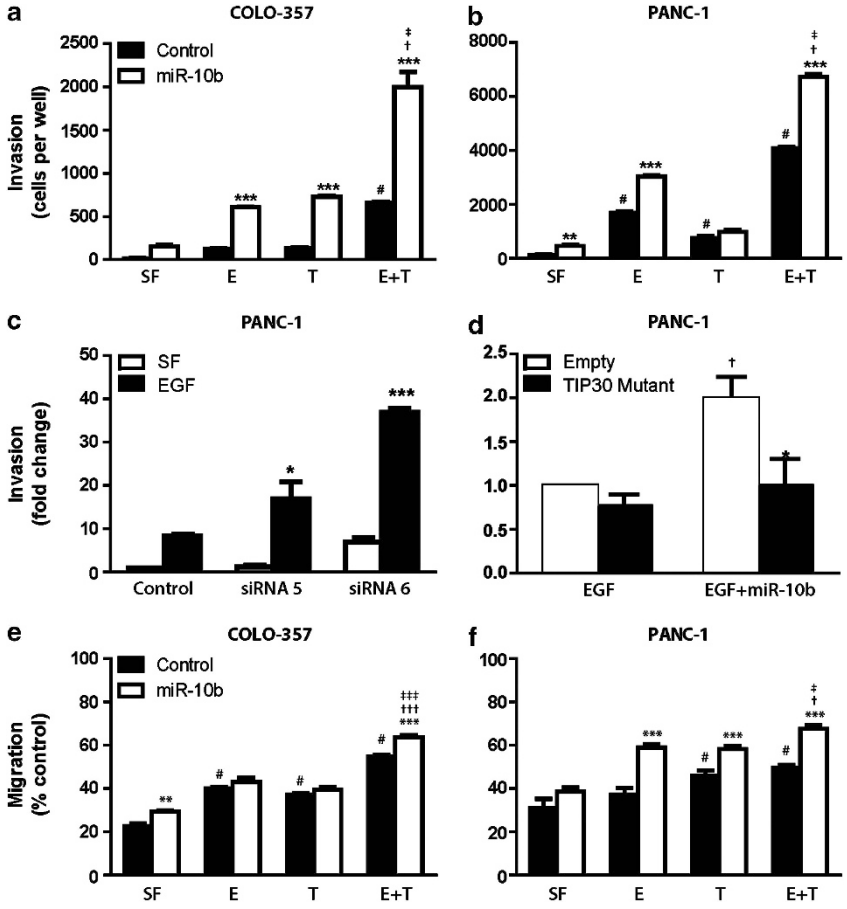

Figure 2. Effects of miR-10b, EGF and TGF-β1 on invasion and migration, and consequences of TIP30 downregulation on invasion. (**a**, **b**) Effects of miR-10b, EGF and TGF-β1 on invasion. COLO-357 (**a**) and PANC-1 (**b**) cells were transfected with control (solid bars) or miR-10b (open bars) precursors and plated (5×10^4 cells/well) in a Matrigel-coated Boyden chamber. Invasion was determined after incubating cells for 20 h in the absence (serum free: SF) or presence of EGF (E, 1 nM), TGF-β1 (T, 0.5 nM) or both EGF and TGF-β1 (E + T). Cells that had invaded across the membrane were stained and counted. (**c**) TIP30 silencing. PANC-1 cells were transfected with scrambled control or two siRNAs (5 and 6) targeting TIP30. Cells (5×10^4 cells per well) were then plated for Matrigel invasion assays in the absence or presence of EGF (1 nM) for 20 h. (**d**) Effects of mutant TIP30 on miR-10b-enhanced invasion. PANC-1 cells were transfected for 48 h with control or precursor miR-10b in combination with either empty pCMV-SPORT6 (Empty) or pCMV-SPORT6-TIP30 carrying a mutated TIP30 cDNA(TIP30 Mutant) that is resistant to miR-10b-induced downregulation. Effects on invasion by 1 nM EGF were then determined. Data in each panel are the means ± s.e.m. from three experiments and were analyzed by two-way ANOVA followed by Bonferroni's adjustment. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with respective controls; $^{\#}P<0.05$, compared with corresponding control SF; $^{\dagger}P<0.01$ compared with EGF alone and $^{\ddagger}P<0.01$ compared with TGF-β1 alone in cells transfected with miR-10b precursor; $^{\dagger}P<0.05$ compared with empty pCMV-SPORT6, and $^{*}P<0.05$ compared with pCMV-SPORT6-TIP30 in miR-10b-transfected group. (**e**, **f**) Migration assays. COLO-357 (**e**) and PANC-1 (**f**) cells were transfected with control (solid bars) or miR-10b precursors (open bars). Cells (5×10^4 cells/well) were then used in a wound-healing assay and incubated for 20 h in the absence (SF) or presence of EGF (E, 1 nM), TGF-β1 (T, 0.5 nM) or both EGF and TGF-β1 (E + T). The wound area was measured at 0 and 20 h using ImageJ software (NIH, Bethedsa, MD, USA). Migration was calculated as the percentage of change in wound area. Two-way ANOVA was performed followed by Bonferroni's adjustment. $^{**}P<0.01$ and $^{***}P<0.001$ compared with respective controls; $^{\#}P<0.001$ compared with control SF; $^{\dagger}P<0.05$ and $^{\dagger\dagger\dagger}P<0.001$ compared with EGF alone in cells transduced with miR-10b; $^{\ddagger}P<0.05$ and $^{\ddagger\ddagger\ddagger}P<0.001$ compared with TGF-β1 alone in cells transduced with miR-10b.

KLF4, *SDC1* and *NF1* expression was decreased by miR-10b in all the three cell lines (Figure 5a), whereas NF2 expression was not altered (Figure 5a). Moreover, miR-10b did not alter the expression of either HOXD10 or TIAM1 (not shown).

We next sought to determine whether TIP30 was expressed in human PDAC samples using the same highly specific antibody that was used in the immunoblotting experiments. TIP30 was readily visible in the cytoplasm of many cancer cells but not in the adjoining stroma. Moreover, high TIP30 expression was principally seen in well to moderately differentiated PDAC (Figure 5b), whereas low TIP30 expression was seen in poorly differentiated PDAC (Figure 5c).

To validate the contribution of miR-10b to PDAC biological aggressiveness, we carried out an *in vivo* study in an orthotopic model using athymic mice (Figure 6) and T3M4 cells engineered to stably overexpress miR-10b. T3M4 cells were used for these studies as they express high EGFR levels[41] and low miR-10b levels (not shown). Both control and miR-10b-overexpressing T3M4 cells formed intra-pancreatic tumors in all mice, but the presence of high levels of miR-10b resulted in faster growing and larger tumors (Figures 6a and b). Although the two tumor groups were similar histologically, miR-10b-overexpressing tumors exhibited many Ki67-positive cells reflecting the presence of a large number of cells in late G1, S, G2 or M phase (Figure 6c). Moreover, there was a marked increase in the number of phospho-histone H3-positive cancer cells that are undergoing mitosis in the miR-10b-overexpressing tumors by comparison with control tumors (Figure 6c). Thus, miR-10b exerts multiple deleterious

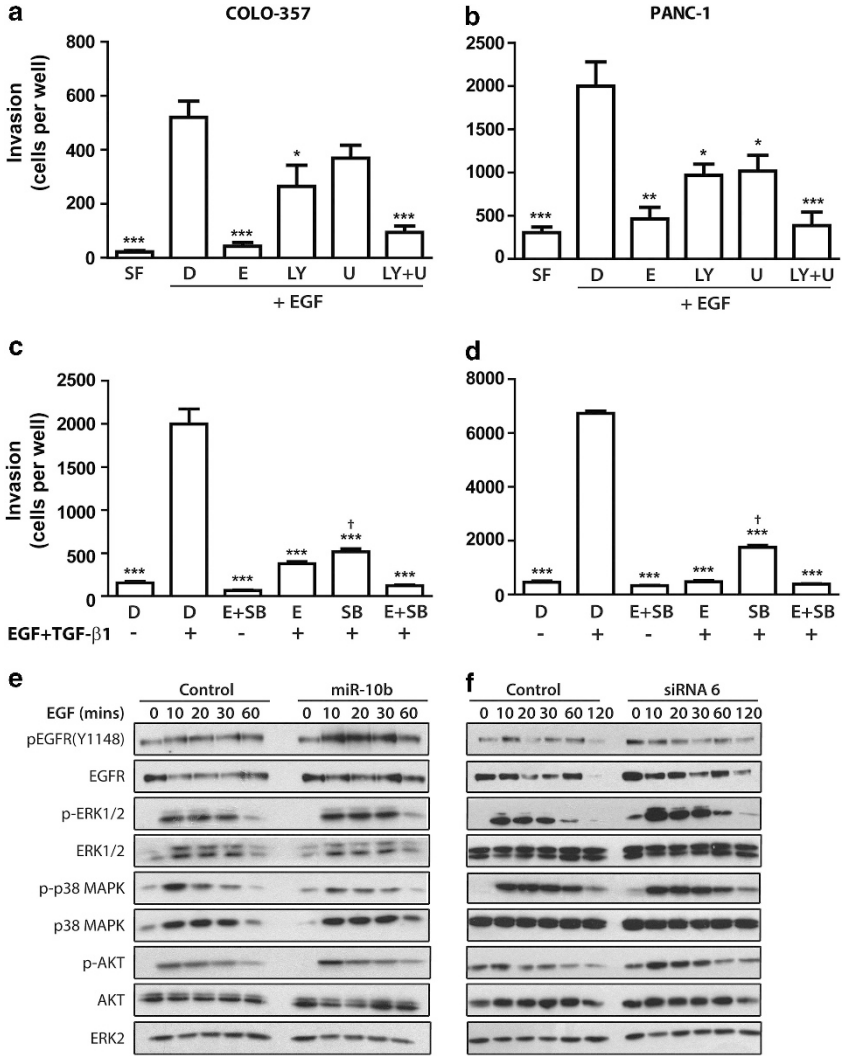

Figure 3. Effects of signaling inhibitors on invasion and effects of miR-10b on signaling. (**a**, **b**) Effects of EGFR inhibitor erlotinib and downstream inhibitors on invasion. COLO-357 (**a**) and PANC-1 (**b**) cells were transfected with miR-10b precursor were incubated for 20 h without EGF (SF) or with EGF (1 nM) in the absence (D: DMSO) or presence of 2 μM erlotinib (E), 10 μM LY294002 (LY: PI3K inhibitor) or 1 μM UO126 (U: MEK inhibitor). (**c**, **d**) Effects of erlotinib and TβRI inhibition with SB505124 on invasion. COLO-357 (**c**) and PANC-1 (**d**) cells transfected with miR-10b precursor were incubated for 20 h without growth factor (−) or with 1 nM EGF and 0.5 nM TGF-β1 (+) in the absence (D: DMSO) or presence of erlotinib (E, 2 μM) or SB505124 (SB, 10 μM), and effects on invasion were determined. Data in all panels are the means ± s.e.m. from three experiments and were analyzed by ANOVA followed by Bonferroni's adjustment. In panels (**a**) and (**b**), $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with EGF alone; in panels (**c**) and (**d**) $^{***}P<0.001$ compared with EGF and TGF-β1 combination and $^{\dagger}P<0.05$ compared with corresponding serum free (SF) values. (**e**, **f**) Effects of miR-10b overexpression and TIP30 knockdown on EGFR signaling. (**e**) PANC-1 cells transfected with control or miR-10b precursor were incubated in the absence of presence of 1 nM EGF for the indicated times. Immunoblotting for the indicated proteins was then carried out. ERK2 was used to assess loading of lanes. (**f**) Effects of TIP30 silencing on EGFR signaling. PANC-1 cells transfected with control siRNA or siRNA targeting TIP30 were incubated in the absence or presence of 1 nM EGF for the indicated times. Immunoblotting for the indicated proteins was then carried out, ERK2 serving to assess loading of lanes.

actions in PCCs, which include upregulation of EGFR signaling, downregulation of NF-1 and decreased expression of metastasis-suppressing genes, thereby contributing to increased PCC proliferation and invasion (Figure 6d).

DISCUSSION

miR-10b was initially reported as a metastasis promoter in breast cancer[27] and subsequently implicated in enhancing metastasis in cancers of the esophagus,[37] colon,[42] liver[43] and pancreas[24] and invasion in glioblastoma.[38] In breast cancer, miR-10b downregulates HOXD10 leading to the increased expression of the metastasis-promoting RhoC.[27] Moreover, in a syngeneic orthotopic mouse model of breast cancer, miR-10b antagomirs significantly attenuated the frequency of metastases to the lungs.[28]

In the present study, we determined that plasma miR-10b levels are markedly increased in PDAC patients by comparison with either normal subjects or patients with chronic pancreatitis, suggesting that assaying for miR-10b could potentially be a useful diagnostic biomarker. Moreover, miR-10b overexpression in PCCs enhanced EGF-stimulated invasion. By gene profiling, immunoblotting and luciferase reporter assays, TIP30 was identified as a target directly downregulated by miR-10b overexpression. Four lines of evidence suggested that miR-10b promoted EGF-mediated invasion by downregulating TIP30 and enhancing EGFR signaling. First, the expression of a TIP30 cDNA resistant to

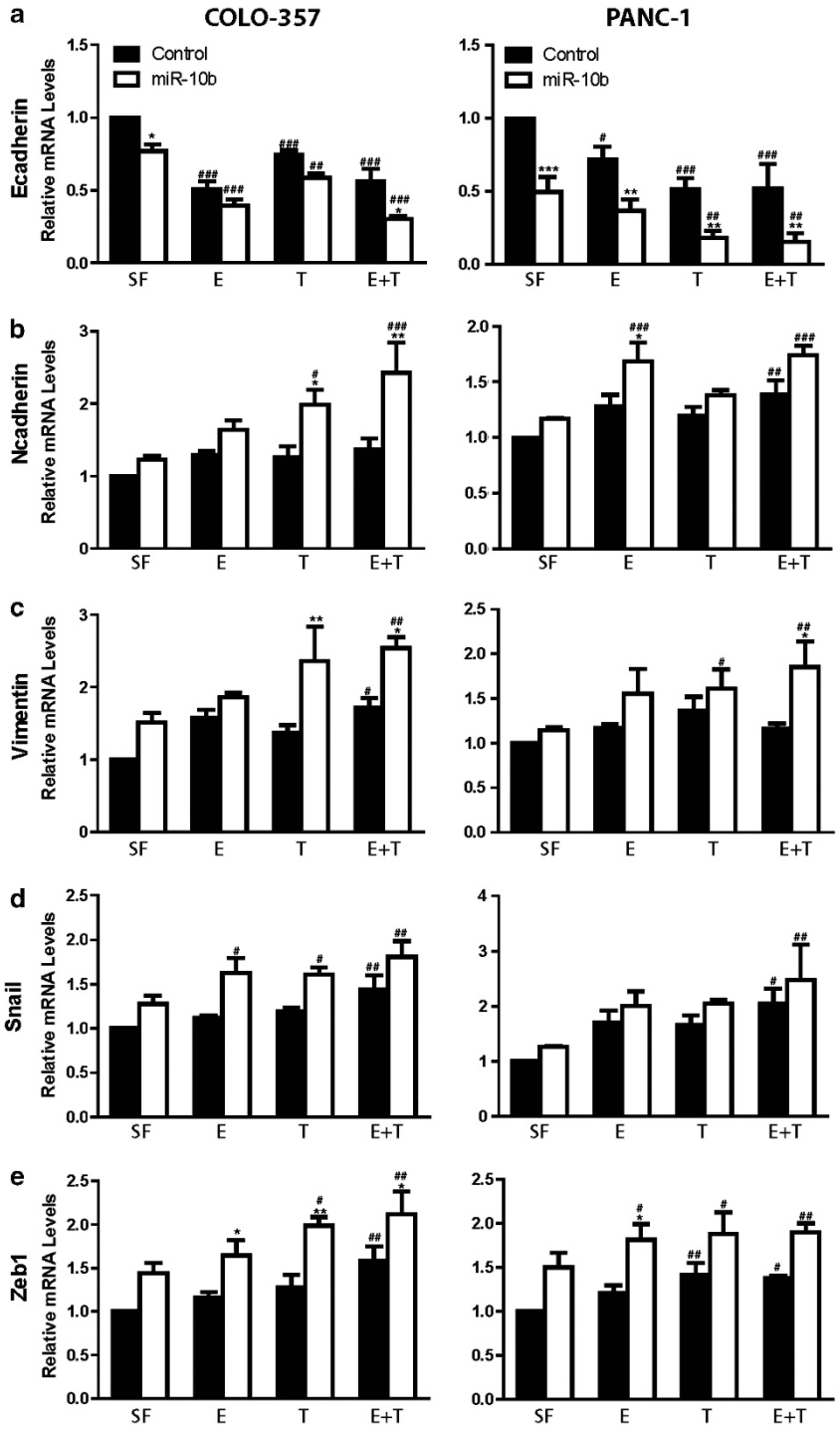

Figure 4. Effects of miR-10b on expression of genes implicated in EMT. (**a**, **e**) qRT–PCR. Control (solid bars) or miR-10b precursor (open bars) transfected COLO-357 and PANC-1 cells were incubated in the absence (serum free: SF) or presence of EGF (E, 1 nM), TGF-β1 (T, 0.5 nM) or both EGF and TGF-β1 (E + T) for 24 h and then harvested for RNA extraction and qRT–PCR for E-cadherin (**a**), N-cadherin (**b**), Vimentin (**c**), Snail (**d**) and Zeb1 (**e**). Data are the means ± s.e.m. from four experiments. Two-way ANOVA was performed followed by Bonferroni's adjustment. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with respective control transfected cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ compared with respective cells in SF condition.

silencing by miR-10b abrogated miR-10b actions on invasion. Second, siRNA-mediated silencing of TIP30 resulted in increased EGF-mediated invasiveness. Third, both high miR-10b levels and TIP30 knockdown enhanced EGF-induced EGFR tyrosine phosphorylation and ERK phosphorylation, whereas attenuating EGFR downregulation. Fourth, EGF-mediated invasion in the presence of high miR-10b levels was blocked by erlotinib and by the combined actions of the PI3K inhibitor LY294002 and the MEK inhibitor UO126, two key pathways downstream of EGFR activation.

PCCs and PDACs express high levels of EGFR, a known mediator of mitogenic signals in general and a promoter of PCC

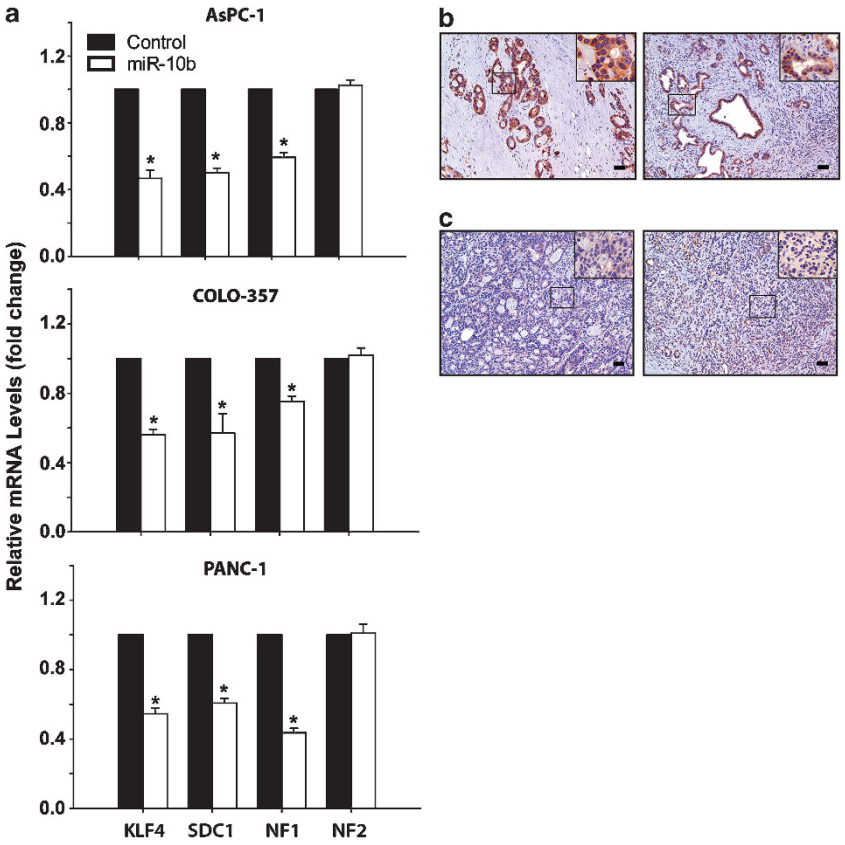

Figure 5. miR-10b targets in human PCCs and TIP30 expression in PDAC. (**a**) qRT–PCR. AsPC-1, COLO-357 and PANC-1 cells were transfected with control (solid bars) or miR-10b precursor (open bars) for 48 h and then harvested for RNA extraction and qRT–PCR for the indicated mRNAs. (**b**, **c**) TIP30 expression in PDAC. Immunohistochemistry for TIP30 in human PDAC tissues shows that TIP30 exhibits strong immunoreactivity in the PCCs in well to moderately differentiated cancers (**b**). By contrast, the PCCs in poorly differentiated PDACs exhibit weak TIP30 immunoreactivity (**c**). Insets show magnified images of boxed areas. Representative images are shown. Bars = 50 μm. *$P < 0.05$, compared with respective control transfected cells.

invasion and metastasis that is essential for PDAC initiation and progression.[44–46] PDACs also express high levels of human EGFR 2 (HER2) and HER3, which heterodimerize with EGFR thereby diversifying and amplifying signaling cascades.[47,48] Moreover, PDACs express high levels of TGF-βs, 14-3-3sigma, MUC1, β1-integrins and delta-N p63, all of which enhance EGFR's ability to activate aberrant pathways that contribute to the invasiveness of PCCs.[26,33,49–52] In the present study, we also determined that TGF-β increased EGF-mediated cell invasion and that this effect was markedly enhanced by high levels of miR-10b. Impressively, the combination of EGF, TGF-β and miR-10b induced a marked increase in cancer cell invasion and EMT-like alterations in gene expression. Thus, miR-10b was facilitating deleterious cross-talk between EGF and TGF-β in a manner that promotes PCC invasion. Given that miR-10b, EGFR and TGF-β are often overexpressed in PDAC,[22–26] these observations suggest that suppressing miR-10b may prevent invasion and possibly metastasis in PDAC while interrupting deleterious EGF–TGF-β interactions that have the potential to contribute to PCC proliferation *in vivo*.

EGF binding to EGFR activates multiple signaling pathways in PCCs, such as the Ras/Raf/MAPK and Rac/JNK/MAPK-38,[51] and EGF then undergoes rapid endocytosis.[53] The EGF/EGFR complexes initially enter into the early endosomes where the EGFR kinase is still active[54,55] and traffic to the late endosome before entering the lysosome where EGFR undergoes degradation.[56] TIP30 accelerates EGFR progression from early-to-late endosomes, whereas TIP30 silencing causes the retention of EGF–EGFR complexes in the early endosome thereby attenuating EGFR degradation and prolonging its signaling.[31,32,57] Inasmuch as PDAC is associated with the overexpression of multiple members of the EGF family, including TGF-α, heparin-binding EGF-like growth factor, amphiregulin and betacellulin,[44] our findings raise the possibility that loss of TIP30 in PDAC may lead to EGFR upregulation in the face of high levels of EGFR ligands that would otherwise promote EGFR degradation. Moreover, loss of TIP30 enhances susceptibility to tumorigenesis[29] and has been associated with metastatic breast cancer.[58] Taken together, these observations suggest that increased miR-10b in PDAC may promote PCC proliferation and metastasis and increase disease aggressiveness by suppressing TIP30 and upregulating EGFR. In support of this conclusion, lower TIP30 levels were associated with a more poorly differentiated histology, and T3M4 cells transduced to overexpress miR-10b exhibited accelerated PCC proliferation and intra-pancreatic tumor growth.

miR-10b promotes invasion in several cancer types by suppressing the expression of various suppressors of metastasis. In breast cancer, in addition to targeting HOXD10, miR-10b enhances invasion by targeting syndecan-1 (SDC1), which is known to suppress metastases.[39] Moreover, miR-10b may act by suppressing KLF4, a zinc finger transcription factor which can also suppress metastasis formation,[37,59] and NF-1,[40] which attenuates ras activity. In the present study, miR-10b did not alter HOXD10 or TIAM-1 expression, but it decreased the expression of SDC1, NF1 and KLF4. This selective targeting suggests that additional factors may bind to the non-targeted mRNA moieties in PCCs, rendering their sites inaccessible to miR-10b, as has been reported for miR-34a.[60]

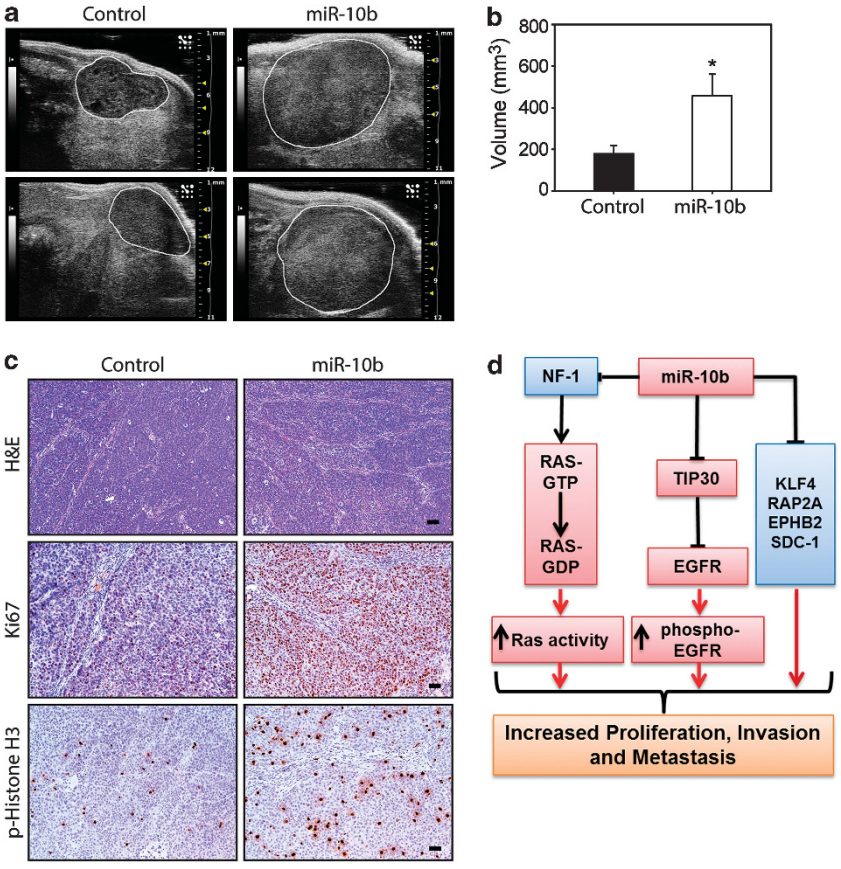

Figure 6. miR-10b overexpression enhances tumor growth. (**a**, **b**) Effects of miR-10b overexpression on T3M4 growth *in vivo*. (**a**) High-resolution ultrasound images obtained 3 weeks post-PCC injection into the pancreas show that compared with cells transduced with an empty vector (control), miR-10b transduction dramatically enhances tumor growth. Tumors are outlined and shown are representative images from two of five control or miR-10b-overexpressing tumors. (**b**) Quantitation of these tumors, calculated via three-dimensional abdominal imaging, shows that miR-10b overexpression (open bar) significantly enhances tumor volume. Data are the means ± s.e.m. for all five control (Sham) and miR-10b-transduced tumors. A *t*-test was used to test for significant differences. $^*P < 0.05$. (**c**) Effects of miR-10b overexpression on proliferation *in vivo*. Immunohistochemistry of control and miR-10b-transduced tumors shows that miR-10b overexpression enhances proliferation as evidenced by the increased Ki67 and phospho-Histone H3 immunoreactivity when compared with control tumors. Shown are representative H&E stains, and Ki67 and phospho-Histone H3 immunostaining from one of five control or miR-10b-transduced tumors. Bars = 50 μm. (**d**) Schematic representation of miR-10b actions in PCCs.

Gene profiling identified two new potential targets downregulated by miR-10b, RAP2A and EPHB2. Bioinformatic analysis predicted that both genes harbor at least one miR-10b binding site, suggesting that they are direct targets of miR-10b. RAP2A has been implicated in cell adhesion and is induced by LKB1, whose loss of function is associated with altered cell polarity and enhanced metastases in lung cancer.[61,62] Similarly, loss of EPHB2 has been associated with increased metastasis in colorectal and gastric cancer.[63,64] miR-10b also enhanced EGF and TGF-β-induced expression of EMT-associated genes decreasing E-cadherin levels, whereas increasing N-cadherin, vimentin, Twist, Snail and Zeb1 levels. Together, these findings suggest that miR-10b modulates a repertoire of genes in PCCs whose downregulation enhances the metastatic process while promoting EMT, which further increases the motility and metastatic potential of these cells.

A number of reports have pointed to reciprocal modulatory interactions between specific miRs and members of the EGF receptor family. Thus, EGFR activation leads to increased expression of miR-21,[65] miR-221 and miR-222.[66] Conversely, miR-7 and miR-133b suppress EGFR expression,[67–69] whereas miR-125a and 125b suppress HER2/HER3, and miR-205 targets HER3 but not other HERs.[70,71] To our knowledge, this is the first study showing the potential connection between miR-10b and EGFR signaling.

Our findings also demonstrate that miR-10b facilitates potentially deleterious interactions between EGFR and TGF-β pathways. Although the exact mechanisms whereby these interactions are enhanced by miR-10b are not known, there are several potentially complementary possibilities. First, TGF-β receptors undergo endocytosis and recycling, and their enhanced retention in the early endosome can increase TGF-β receptor signaling.[72] It is therefore possible that co-retention in the early endosome may facilitate cross-talks between these receptors. Second, both MAPK activation and Smad signaling are essential for TGF-β-induced EMT,[73] and miR-10b enhanced EGF's ability to activate MAPK. Third, within the endosome, EGFR induces AGO2 phosphorylation to AGO2-Y393, thereby reducing AGO2 binding to Dicer and attenuating the processing of precursor miRNAs to mature miRNAs.[74,75] By contrast, TGF-β promotes Drosha-mediated miRNA maturation[76,77] and enhances the expression of EMT-inducing miRs.[35] These observations raise the possibility that TGF-β may act, in part, by interfering with EGFR's actions on Dicer.

In summary, our findings indicate that circulating miR-10b levels could serve as a diagnostic biomarker in PDAC and suggest that targeting miR-10b may represent an important therapeutic approach in this deadly cancer that could reverse the multiple deleterious actions of this oncomir.

MATERIALS AND METHODS

Cell culture

ASPC-1 and PANC-1 human PCCs were obtained from ATCC (Manassas, VA, USA), which validated their authenticity. COLO-357 and T3M4 human PCCs were a gift from Dr RS Metzger at the Duke University. Their authenticity was validated by chromosomal analysis. ASPC-1 cells were grown in RPMI 1640, and PANC-1 and COLO-357 cells were grown in Dulbecco's modified Eagle's medium. Media were supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) at 37 °C in humidified 5% CO_2 incubator. Serum-free medium was supplemented with 0.1% bovine serum albumin, 5 μg/ml apotransferrin and 5 ng/ml sodium selenite. EGF (Millipore, Billerica, MA, USA) and TGF-β (Genentech, Inc., South San Francisco, CA, USA) were added at the indicated concentrations.

RNA isolation and quantitation

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Assaying of mature miRNAs was performed using the TaqMan miRNA qRT–PCR kit (Life Technologies, Gaithersburg, MD, USA) using RNU6B as an internal control. To assay mRNAs, RNA (500 ng) was reverse transcribed to cDNA with a high-capacity RNA-to-cDNA master mix, and qRT–PCR was performed with Power SYBR Green Master Mix (both from Life Technologies). Primers were designed using the Prime-BLAST online tool.

Transfection

Cells were transfected with 20–60 nM miR-10b precursor (known as pre-miR hsa-miR-10b precursor, sense: 5′-UACCCUGUAGAACCGAAUUUGUG-3′, Life Technologies) or a control precursor (known as pre-miR non-targeting negative control, sense: 5′-UGUACUGCUUACGAUUCGGTT-3′) at 50% confluency. Transfected cells were plated for MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (24 h post transfection) or transwell invasion assays (48 h post transfection). Non-targeting siRNA or siRNAs (ON-TARGET*plus* siRNA, Dharmacon, Lafayette, CO, USA) designed to target TIP30 were used to silence TIP30 in COLO-357 (60 nM) and PANC-1 (20 nM) cells. For TIP30 rescue experiments, PANC-1 cells were transfected with 20 nM miR precursor and 250 ng of pCMV-SPORT6-TIP30 cDNA and Lipofectamine 2000 (both from Invitrogen), which was used in all transfection studies.

Transduction

To stably overexpress miR-10b in PCCs, the MDH1-PGK-GFP microRNA-10b retroviral construct.[28] Addgene plasmid No. 16070) or an empty MDH1-PGK-GFP construct (Addgene plasmid No. 11375) were transfected into Phoenix cells, a 293 T-based retroviral packaging cell line (Life Technologies). Viruses were harvested, and PCCs were transduced as described.[78] After 48 h, GFP-positive cells PCCs were isolated (Flow Cytometry Facility, Indiana University School of Medicine, Indianapolis, IN, USA), re-plated and cultured as described above.

MTT, invasion and migration assays

MTT assays were performed in 96-well plates.[49] miR-10b precursor and precursor control transfected cells (5×10^4) were plated in Matrigel pre-coated transwell chambers (BD Biosciences, San Jose, CA, USA), and invasion assays were performed as previously reported.[49,78] UO126 (1 μM) were from Alexis Biochemicals (San Diego, CA, USA). Erlotinib (2 μM; Genentech, Inc.) and LY294002 (10 μM; Calbiochem, San Diego, CA, USA) were added to serum-free medium in the lower chamber in the absence or presence of 1 nM EGF and/or 0.5 nM TGF-β1. Wound-healing assays were performed as previously reported[49,78] 24 h after cell transfection with pre-miR-10b oligonucleotides.

Microarray and data analysis

miR-10b precursor or control transfected PANC-1 cells were harvested at 20 h post transfection. Total RNA (three replicates per condition) was extracted using TRIZOL reagent. Microarray analysis for microRNAs was performed by the Genomics and Microarray Core Facility at Dartmouth Medical School. Array data were registered with GEO (accession No. GSE40189) for public access.

Immunoblotting

Whole-cell extracts were prepared in ice-cold lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA (ethylene glycol tetraacetic acid), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF and one tablet of complete protease inhibitor/10 ml. Lysates (25 μg/lane) were subjected to 10-12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to PVDF (polyvinylidene difluoride; Millipore) and immunoblotted.[33] Antibodies directed against the following antigens were used: EGFR, Phospho-EGFR Tyr1148, phospho-AKT Ser473, AKT, phospho-p44/42 MAPK Thr202/Tyr204, p44/42 MAPK, phospho-p38 MAPK Thr180/Tyr182, p38 MAPK (all from Cell Signaling Technology, Danvers, MA, USA), and ERK2 (C-14, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The anti-TIP30 antibody was a kind gift from Dr Hua Xiao at the Michigan State University.

Constructs

The LightSwitch TIP30 3′UTR Reporter (Switchgear Genomics, Menlo Park, CA, USA) was utilized to assess whether there is a direct interaction between miR-10b and TIP30 3′UTR. Site-directed mutagenesis kits (Stratagene, La Jolla, CA, USA) were used to introduce a six-base pair mutation in the seed-binding site of TIP30 3′UTR. The pCMV-SPORT6 TIP30 cDNA construct used in the TIP30 rescue experiment was purchased from Open Biosystems, Huntsville, AL, USA.

Luciferase reporter assay

Cells in 24-well plates (50% confluency) were co-transfected with miR-10b precursor (20 nM) and TIP30 3′UTR Reporter (200 ng). Luciferase activity was measured 24 h later using the Dual-Glo luciferase assay system (Promega, Fitchburg, WI, USA).

Circulating miR-10b quantitation

Plasma from 20 normal, 5 chronic pancreatitis and 17 PDAC patients was obtained from the Indiana University Simon Cancer Center Solid Tissue Bank (Indianapolis, IN, USA). RNA was isolated from 100 μl of plasma using Trizol LS (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. qRT–PCR for miR-10b was performed in duplicate with a Taqman micro RNA reverse transcription kit and a miR-10b-specific primer/probe set (Life Technologies). Ct values were obtained using a Viia 7 Real-time PCR System (Life Technologies). miR-10b levels were normalized to miR-16 levels, a stable and abundant circulating microRNA[79] that is a suitable endogenous control for analyzing circulating microRNAs in PDAC plasma.[80] Fold increases were calculated relative to miR-10b levels in normal plasma.

Orthotopic model

Sham- and miR-10b transduced T3M4 cells were injected into the tail region of each pancreas (500.000 cells/injection) of five 8-week-old male athymic mice. Tumors were imaged 3 weeks later with a Vevo2100 high resolution ultrasound (Visual Sonics Inc., Toronto, ON, Canada). Tumor volumes were calculated using the three-dimensional abdominal images and the Vevo2100 System software. Tumors were harvested, fixed, embedded, sectioned and hematoxylin and eosin stained.[81]

Immunohistochemistry

Paraffin-embedded human PDAC tissues were obtained from the Indiana University Simon Cancer Center Solid Tissue Bank, and 5-μm sections were stained for TIP30 using the anti-TIP30 antibody as described.[82] For the orthotopic tumors, immunohistochemistry was performed on 5-μm sections using Ki67 (Novocastra, UK) and phospho-Histone H3 (Cell Signaling Technology, Inc.) antibodies.[81,82]

Statistical analysis

Results are presented as mean ± s.e.m. Statistical differences between groups were assessed using one sample *t*-test analysis, one-way or two-way ANOVA, as indicated, followed by Bonferroni post-test analysis. $P < 0.05$ was considered as statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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